Emergence of *Citrobacter freundii* carrying IMP-8 metallo-β-lactamase in Germany

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Abstract

Metallo-β-lactamases (MBLs) in *Enterobacteriaceae* are an increasing problem worldwide. This report describes the isolation of *Citrobacter freundii* carrying IMP-8 MBL from three patients during the period from March 2012 until March 2013 in Germany. The bla_{IMP-8} enzyme is predominantly found in Asia, where IMP-8 has spread to various enterobacterial species causing serious infections. To our best knowledge, this is the first report of bla_{IMP-8} habouring *Enterobacteriaceae* in Europe.

Keywords: Antimicrobial resistance, IMP, infection control, laboratory surveillance, metallo-β-lactamases, multidrug resistance
Original Submission: 3 October 2013; **Revised Submission:** 22 December 2013; **Accepted:** 14 January 2014
Article published online: 21 March 2014
New Microbe New Infect 2014; **2:** 42–45

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Introduction

The emergence and spread of carbapenemase-producing *Enterobacteriaceae* is an increasing problem of global dimensions. Originally, metallo-β-lactamases (MBLs) were associated with resistance in Gram-negative non-fermenters, but they have become increasingly important regarding carbapenem resistance in *Enterobacteriaceae*. MBLs confer resistance to almost all β-lactam antibiotics and are not inactivated by β-lactamase inhibitors, hence limiting treatment options in the individual patient and presenting a major challenge for infection control within the hospital setting [1].

To date, the occurrence of carbapenem-resistant *Enterobacteriaceae* in Germany is still a rare event; however, outbreaks involving KPC and VIM-1 carrying *Klebsiella pneumoniae* isolates have been described [2,3]. In the year 2012, VIM-1 was the most prevalent MBL detected in *Enterobacteriaceae* in Germany, followed by NDM-1 and GIM-1 [2]. IMP type MBLs were first identified in *Pseudomonas aeruginosa* in Japan [4] and have since been reported predominantly from Asia [5]. With the exception of Italy, IMP-type enzymes have rarely been reported from other countries in Europe [1,5,6].

Here, we report the isolation of *Citrobacter freundii* harbouring MBL IMP-8 from three patients between March 2012 and March 2013. All isolates were obtained from rectal swabs. All three patients had underlying haematological conditions (acute myeloid leukaemia $n = 2$ and myelodysplastic syndrome $n = 1$) and underwent haematopoietic stem cell transplantation. They were screened from rectal swabs for colonization with multidrug resistant Gram-negative bacteria on a weekly routine schedule.

Laboratory Analysis

Identification of the isolates was performed using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF-MS) (AXIMA Assurance, bioMérieux SA, Marcy l'Etoile, France; Saramis Database Version 4.09) and the VITEK 2 identification system (bioMérieux SA). Antimicrobial susceptibility testing was initially performed with the VITEK 2 system (bioMérieux SA) and confirmed by Etest (bioMérieux SA). Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_3.1.pdf). The detection of extended-spectrum β-lactamase genes bla_{CTX-M}, bla_{TEM}, bla_{SHV}, [7] and carbapenemase genes bla_{OXA-48} [8], bla_{KPC} [9], bla_{NDM} [10], bla_{IMP} and bla_{VIM} [11] was performed as described previously. Sequencing of the IMP genes was performed using class 1 integron primer 3CS (5′-AAG CAG ACT TGA CCT GA-3′) in combination with primer IMP-A, and 5CS (5′-GGC ATC CAA GCA GCA AG-3′) in combination with primer IMP-B [11]. Sequence identification was determined by comparison with the sequences available in GenBank (http://www.ncbi.nih.gov/BLAST) and with the reference sequences of

the Lahey database (http://www.lahey.org/studies). Alignment was performed using BioEdit version 7.1.11 (Ibis Biosciences, Carlsbad, CA, USA). Genomic fingerprinting of the isolates was done by the enterobacterial repetitive intergenic consensus (ERIC) method using the ERIC2 primer as described previously [12]. Plasmids were extracted using the Qiagen large construct kit (Qiagen, Hilden, Germany) and digested using *Eco*RI and *Bam*HI to allow for size estimation of the plasmids. Southern blotting was performed following a standard protocol. Briefly, DNA was blotted onto positively charged nylon membranes (Roche Biochemicals, Basel, Switzerland) in denaturation solution (3 M NaCl, 0.4 M NaOH) by capillary transfer. High stringency hybridization was performed in accordance with the instructions given by the manufacturer of the digoxigenin labelling and detection kit (Roche Biochemicals). Digoxigenin-labelled DNA probes were generated with a digoxigenin-labelling PCR kit as described in the manufacturer's instructions (Roche Biochemicals) using the oligonucleotides IMP-A and IMP-B.

Results

The characteristics of the isolates are summarized in Table 1. All three isolates were resistant to piperacillin-tazobactam, cefuroxime and cefotaxime. The MIC of meropenem was >32 mg/L for the isolate of patients two and three, whereas meropenem MIC for patient one was intermediate with an MIC of 8 mg/L. All three strains were susceptible to tigecycline, colistin and amikacin. Molecular detection of ESBL genes bla_{CTX-M}, bla_{TEM}, bla_{SHV} and carbapenemase genes bla_{OXA-48}, bla_{KPC}, bla_{NDM} and bla_{VIM} was negative in all three isolates. The IMP PCR gave a positive result and subsequent determination of the nucleotide sequence revealed an MBL of the IMP-8 type. The sequence was compared with the reference sequence (GenBank accession number AF322577) [13]. The bla_{IMP-8} gene detected in the three isolates harboured a non-coding point mutation at position 18 (T→C) as shown in Fig. 1. Additionally, an IMP-8 carrying plasmid of approximately

TABLE 1. Characteristics of IMP-8 carrying *Citrobacter freundii* isolates from rectal swabs of three hospitalized patients in Germany

			Etest results: MIC for the antimicrobial agents in mg/L and interpretation[a]											
Patient	Source	Isolation date	TZP	CXM	CTX	CAZ	FEP	ATM	ERT	MEM	TIG	CIP	AN	COL
1	Rectal swab	March 2012	128 R	>256 R	16 R	>256 R	16 R	2 I	>32 R	8 I	0.75 S	8 R	2 S	0.75 S
2	Rectal swab	June 2012	96 R	>256 R	16 R	>256 R	24 R	3 I	>32 R	>32 R	0.75 S	16 R	2 S	1 S
3	Rectal swab	March 2013	>256 R	>256 R	24 R	>256 R	24 R	2 I	>32 R	>32 R	0.75 S	12 R	2 S	1 S

I, intermediate; R, resistant; S, susceptible; AN, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; COL, colistin; CTX, cefotaxime; CXM, cefuroxime; ERT, ertapenem; MEM, meropenem; TIG, tigecycline; TZP, piperacillin-tazobactam.
[a]Interpretation according to the EUCAST clinical breakpoints (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_3.1.pdf).

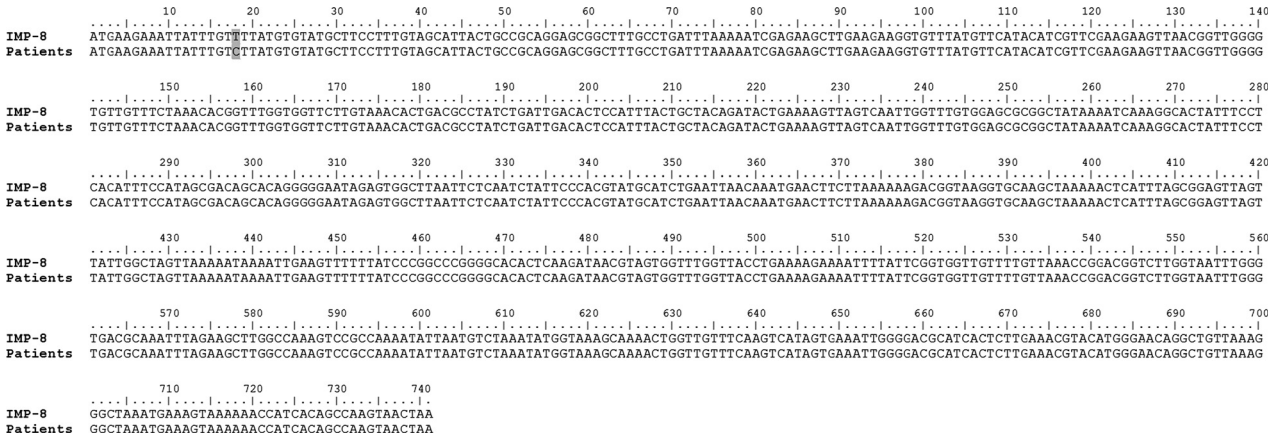

FIG. 1. Sequence of the bla_{IMP-8} gene of three *Citrobacter freundii* strains isolated from hospitalized patients in Germany. Compared with the sequence of the IMP-8 reference strain (GenBank accession number AF322577 [13]), a non-coding point mutation at position 18 was identified in the IMP-8 from the three *Citrobacter freundii* strains (grey shaded).

28 500 bp could be isolated from all *C. freundii* strains. Genomic fingerprinting by the ERIC method revealed indistinguishable PCR patterns in all isolates (data not shown).

Conclusion

This is the first report of IMP-8 MBL in *Enterobacteriaceae* in Germany. IMP-8 is very uncommon in Europe, only once reported from Portugal in a *Pseudomonas mendocina* strain [6, 14]. In contrast, IMP-8 is frequently encountered in Asia, especially in Taiwan, where IMP-8-producing *Enterobacteriaceae* are involved in serious infections. Yan *et al.* reported on a case series of 37 patients with bloodstream infections caused by a large variety of IMP-8-producing enterobacterial species including *Escherichia coli*, *K. pneumoniae*, *Enterobacter cloacae* and *C. freundii* [15]. Furthermore, aggravating the issue, phenotypic screening for IMP-8-positive *Enterobacteriaceae* is extremely difficult because of the lack of distinctive phenotypes. Investigation of 95 IMP-8-positive *Enterobacteriaceae* revealed susceptibility to ertapenem in 21% and to meropenem in 45%, whereas phenotypic combined disk tests using EDTA and phenylboronic acid were positive in only 40% of the isolates [16]. These observations from Taiwan suggest that IMP-8 is capable of spreading between enterobacterial species, causing serious problems in terms of infection control measures and limiting therapeutic options in critically ill patients.

The role of faecal carriage of MBL-producing *Enterobacteriaceae* remains unclear and has not been investigated in a large-scale epidemiological study. Faecal carriage of one *C. freundii* VIM-1 has been reported from Spain in an outpatient, who was also colonized with two different VIM-1-carrying *K. pneumoniae* isolates [17]. In an Italian hospital, eight VIM-1-carrying *C. freundii* strains were isolated from rectal swab samples during active screening following the detection of a *K. pneumoniae* carbapenemase (KPC) -positive patient [18].

None of our three patients became infected by the IMP-8 *C. freundii* strains, but nevertheless it is alarming that IMP-8 MBL circulates in the gut flora of patients at high risk of nosocomial infections. Even more worrisome is the fact, that the IMP-8 gene is located on a plasmid, which might facilitate the transfer of the resistance gene within enterobacterial species. Our findings emphasize the importance of establishing screening schemes and laboratory diagnostic algorithms to ensure the implementation of efficient infection control measures and therapeutic strategies, not only in high-prevalence countries, but also in countries with a low incidence of MBL producing *Enterobacteriaceae*.

Acknowledgement

This work was supported by the German Centre for Infection Research (DZIF). We thank Nadine Hoffmann for expert technical assistance.

Conflict of Interest

None declared.

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